Process for the preparation of 1,3-propanediol by a recombinant micro-organism in the absence of coenzyme B12 or one of its precursors

This invention relates to a process for the preparation of 1,3-propanediol from a carbon-containing substance, said process comprising a step involving the culture of a recombinant micro-organism not producing coenzyme B12 in the absence of the addition of coenzyme B12 or of one of its precursors.

The invention also relates to a nucleic acid coding for a glycerol dehydratase, the catalytic activity of which is independent of the presence of coenzyme B12 or of one of its precursors as well as a nucleic acid coding for a 1,3-propanol dehydrogenase implicated in the synthesis of 1,3-propanediol.

The invention also relates to recombinant vectors and host cells comprising said nucleic acids as well as the polypeptides encoded by the latter.

1,3-Propanediol is a substance of great industrial importance, used mainly in the industrial production of detergents and polymers.

Thus, 1,3-propanediol is used in liquid detergents as a stabilizing agent for lipases, amylases and proteases as well as "protective softener" in the liquid detergents for dishwashing by hand.

Moreover, 1,3-propanediol is used increasingly in the industrial production of polymers, more particularly as a monomer used to synthesize polyesters, polyethers or polyurethanes.

Currently, the production of 1,3-propanediol is achieved mainly by chemical synthesis involving hydration (in acid medium) of acrolein to 3-hydroxypropionaldehyde, which is then reduced to 1,3-propanediol by catalytic hydrogenation.

Such a process is expensive and makes use of a toxic product, acrolein. Furthermore, this synthesis is poorly selective and generates a large number of unusable by-products.

Another synthetic route consists of hydrocarbonylation of ethylene oxide by carbon monoxide and hydrogen under high pressure in the presence of catalysts and solvents.

Such a reaction produces a dioxane which is then hydrogenated to 1,3-propanediol. This second process of chemical synthesis is also very expensive.

For several years an alternative to the production of 1,3-propanediol by chemical synthesis has been the subject of various studies : the bioconversion of glycerol to 1,3-propanediol by certain bacterial strains such as *Citrobacter, Clostridium, Enterobacter, Klebsiella, Lactobacillus* and *Pelobacter*.

In particular, the conversion of glycerol to 1,3-propanediol in facultative anaerobic bacteria such as bacteria of the *Klebsiella* genus, *Citrobacter* genus or even *Enterobacter agglomerans* has been studied.

Thus, attempts have been made to clone the genes coding for enzymes responsible for the conversion of glycerol to 1,3-propanediol from the bacterium *Klebsiella pneumoniae*.

More particularly, the cloning of the genes coding for two enzymes has been investigated, a glycerol dehydratase which catalyses the conversion of glycerol to 3-hydroxypropionaldehyde and a 1,3-propanediol dehydrogenase which catalyses the conversion of 3-hydroxypropionaldehyde to 1,3-propanediol, respectively.

U.S. patents 5,633,362 and 5,821,092 describe the cloning of a genomic fragment of about 35 kb of the bacterium *Klebsiella pneumoniae*, said DNA fragment containing a sequence coding for an active diol dehydratase. Such a genomic fragment was obtained by screening of several cosmid libraries of the bacterial species *Klebsiella pneumoniae* and *Klebsiella aerogenes*.

It is described in these patents that strains of *E. coli DH5α* were transformed with these cosmids and the bacterial transformants were screened for their capacity to convert glycerol into 1,3-propanediol. A low level of production of 1,3-propanediol was observed in certain clones of bacterial transformants. This led to the deduction that the diol dehydratase encoded by the cosmids selected might be responsible for the conversion of glycerol to 1,3-propanediol observed.

Nonetheless, the production of 1,3-propanediol by the *E. coli DH5α* strains transformed by the cosmids selected necessarily required the

presence in the bacterial culture medium of vitamin B12 or one of its precursors.

Furthermore, the fermentation time necessary for the detection of 1,3-propanediol production was very long (from 78 to 132 hours) and the level of production of 1,3-propanediol was very low.

U.S. patent No. 5,686,276 in the name of DU PONT DE NEMOURS & COMPANY describes a process making possible the bioconversion of D-glucose to 1,3-propanediol by an *E. coli* strain transformed by the cosmid DNA originating from *Klebsiella pneumoniae*. Again, the production of 1,3-propanediol requires the use of culture medias adapted to the *E. coli* cells transformed by a *Klebsiella pneumoniae* cosmid containing vitamin B12, for example at a concentration of 800 µg/ml.

The levels of production of 1,3-propanediol observed were very low, of the order of 0.5 g/l to 10 g/l.

The presence of vitamin B12 in the culture medium of the cells transformed by the *Klebsiella pneumoniae* DNA of the U.S. patents cited above is necessary owing to the fact that the co-enzyme B12 is a necessary cofactor for the catalytic activity of the glycerol dehydratase of *Klebsiella pneumoniae*.

The coenzyme B12 or any of its precursors such as vitamin B12 is an extremely expensive and very unstable substance, and this makes it difficult and even impossible to transfer to an industrial scale the processes for the conversion of glucose or glycerol to 1,3-propanediol by bacterial fermentation with the aid of such strains.

In addition, coenzyme B12 and its precursors pass through the membranes of certain micro-organisms, such as yeasts, with difficulty, and this requires the presence of very high concentrations of these substances in the culture medium in order that they become accessible to the intracellular enzymes of which coenzyme B12 is the cofactor.

Moreover, an alternative which would consist of introducing the DNA coding for the known glycerol dehydratases implicated in the conversion of glycerol to 1,3-propanediol into bacteria synthesizing vitamin B12 encounters considerable technical obstacles.

In fact, only certain bacterial species synthesize vitamin B12 naturally, such as the *Pseudomonas* bacteria or also the propionibacteria,

the genetics of which is very poorly understood and which are therefore not capable of undergoing genetic modifications.

Other bacteria which synthesize vitamin B12 naturally and the genetics of which is well understood such as *Klebsiella pneumoniae* present considerable problems of toxicity which makes them unsuitable for industrial use.

In addition to this first disadvantage it was possible to obtain only a low level of production of 1,3-propanediol after transformation of *Klebsiella pneumoniae*. Thus, the PCT application No. WO 98/21 339 describes recombinant *Klebsiella pneumoniae* expressing both the genes for the metabolism of glucose to glycerol and the genes for the metabolism of glycerol to 1,3-propanediol. The level of production of 1,3-propanediol observed starting from glucose was low, of the order of 10 g per liter.

The technical obstacles to the development of a process for the bioconversion of a carbon-containing substance to 1,3-propanediol mentioned above have been overcome by the invention.

In fact, the applicant has isolated and characterized a nucleic acid which codes for a glycerol dehydratase, the catalytic activity of which is independent of the presence of coenzyme B12 or any one of these precursors.

The genes coding for the coenzyme B12-independent glycerol dehydratase were isolated by the applicant from the genome of the bacterium *Clostridium butyricum* VPI 1718. They code for a dimeric protein constituted of two protein subunits, the polypeptides ORF11 and ORF12, respectively.

It has been shown according to the invention that the sequences coding for the polypeptides ORF11 and ORF12 are located in a unique operon in the *Clostridium butyricum* genome, said operon also comprising downstream from the nucleic acid sequence coding for the ORF12 subunit a region coding for a 1,3-propanediol dehydrogenase.

The operon according to the invention comprises from the 5' end to the 3' end a promoter of transcription, the sequence *orf11* coding for ORF11, the first subunit of the coenzyme B12-independent glycerol dehydratase, the sequence *orf12*, coding for ORF12 the second subunit of

the glycerol dehydratase and a sequence dhaT coding for a novel 1,3-propanediol dehydrogenase (DHAT).

Thus, the nucleic acid sequences coding for the two protein subunits of the glycerol dehydratase and for 1,3-propanediol dehydrogenase form part of a unique operon, the three coding sequences being regulated by a unique promoter sequence situated on the 5' side of the sequence coding for the subunit ORF11.

It has also been shown according to the invention that the transformation of a bacterial cell host by a sequence comprising the regions coding respectively for ORF11, ORF12 and DHAT was such as to provide on the transformed cell host the capacity to produce 1,3-propanediol from glucose or glycerol in the absence of the coenzyme B12 or any of its precursors when these sequences are placed under the control of a suitable promoter that is functional in the cell host in which the expression of these sequences is desired.

The applicant has also shown that the transformation of a bacterial cell host producing 1,3-propanediol naturally by the sequences coding for the two subunits of the glycerol dehydratase according to the invention was such as to induce a significant increase in the production of propanediol in the recombinant bacterial hosts obtained.

The object of the invention is thus a process for the preparation of 1,3-propanediol from a carbon-containing substance, said process comprising at least one step which consists of culturing a recombinant micro-organism in which has been integrated at least one nucleic acid coding for the two subunits of a glycerol dehydratase the catalytic activity of which is independent of the presence of coenzyme B12 or one of its precursors.

Advantageously, the glycerol dehydratase whose catalytic activity is independent of the presence of coenzyme B12 is derived from the bacterium *Clostridium butyricum.*

Preferably, the coenzyme B12-independent glycerol dehydratase is a dimeric protein composed of a first polypeptide having at least 50 % amino acid identity with the polypeptide of sequence SEQ ID No. 6 and a second polypeptide having at least 50 % amino acid identity with the polypeptide of sequence SEQ ID No. 7. Very advantageously, the

coenzyme B12-independent glycerol dehydratase is composed of a first and second polypeptide having an amino acid identity of at least 60 %, 65 %, 70 %, 80 %, 85 %, 90 %, 95 % or even 99 % with the polypeptide of sequence SEQ ID No. 6 and the polypeptide of sequence SEQ ID No. 7, respectively.

The "identity percentage" of nucleotides or amino acids between two sequences in the sense of the present invention can be determined by comparing two optimally aligned sequences, through a window of comparison.

The part of the nucleotide or peptide sequence in the window of comparison may thus include additions or deletions (for example, "gaps") with respect to the reference sequence (which does not comprise these additions or these deletions) so as to obtain an optimal alignment of the two sequences.

The percentage of identity is calculated by determining the number of positions at which an identical nucleotide base or amino acid is observed in the two sequences compared, then by dividing the number of positions at which there is identity between the two bases or the two amino acids by the total number of positions in the window of comparison, then by multiplying the result by a hundred in order to obtain the percentage of sequence identity.

The optimal alignment of the sequences for the comparison can be carried out by computer with the aid of known algorithms (for example, the FASTA software of the company WISCONSIN GENETICS SOFTWARE PACKAGE, GENETICS COMPUTER GROUP (GCG), 575 Science Doctor, Madison, WISCONSIN).

As an illustration, the percentage of sequence identity can be calculated with the aid of the previously mentioned FASTA software, by using exclusively the default parameters.

Very preferably, the percentage of nucleotide or amino acid identity between two nucleic acid or amino acid sequences is calculated with the aid of the BLAST software (version 2.06 of September 1998) by using exclusively the default parameters.

The differences in amino acids that a polypeptide according to the invention may comprise with respect to the reference amino acid sequence,

such as defined in the listing of sequences presented at the end of the present description, may or may not result in substitutions, deletions or additions of one or more consecutive amino acids.

In accordance with another aspect of the process according to the invention, the recombinant micro-organism comprises in addition a nucleic acid coding for a 1,3-propanediol dehydrogenase, preferably a 1,3-propanediol dehydrogenase of *Clostridium butyricum*.

Preferably, the 1,3-propanediol dehydrogenase is a polypeptide having at least 90 % amino acid identity with the polypeptide of sequence SEQ ID No. 8.

Very advantageously, the invention also relates to a polypeptide having at least 95 %, 97 %, 98 % or 99 % amino acid identity with the polypeptide of sequence SEQ ID No. 8.

An essential characteristic of the process according to the invention is that the step involving the culture of the recombinant micro-organism is carried out in the absence of coenzyme B12 or one of its precursors.

In accordance with another aspect, the process is also characterized in that the carbon-containing source is selected from the carbohydrates and the polyols.

The carbohydrate may, for example, be glucose.

The polyol may, for example, be glycerol.

Preferably, the process according to the invention is carried out with a micro-organism selected from the micro-organisms not naturally producing coenzyme B12 or one of its precursors.

Such a micro-organism may be a bacterium belonging to the *Clostridium* or *Escherichia* genus.

It may also be a yeast of the *Saccharomyces cerevisiae* species.

In accordance with a particular embodiment, the process is also characterized in that the recombinant micro-organism also comprises nucleic acids coding respectively for a glycerol-3-phosphate dehydrogenase and a glycerol-3-phosphatase, in which case the recombinant micro-organism is capable of converting a carbon-containing source such as glucose into 1,3-propanediol in high yield.

Another object of the invention is a nucleic acid comprising all or part of a polynucleotide coding for at least one subunit of a glycerol

dehydratase whose catalytic activity is independent of the presence of coenzyme B12 or one of its precursors.

Preferably, a nucleic acid according to the invention is available in a purified or isolated form.

Such a polynucleotide should have preferably at least 50 % nucleotide identity with the polynucleotide of sequences SEQ ID No. 1 or SEQ ID No. 2.

A polynucleotide with a sequence complementary to the polynucleotides of sequences SEQ ID No. 1 or SEQ ID No. 2 also constitutes an object of the invention.

Also included in the invention are nucleic acids comprising all or part of a polynucleotide possessing at least 60 %, 65 %, 70 %, 75 %, 85 %, 90 %, 95 %, 98 %, 99 %, 99.5 % or even 99.8 % of nucleotide identity with the nucleotide sequence of any one of the nucleic acids whose sequences are defined in the present description, or a nucleic acid with a complementary sequence.

The term "isolated" in the sense of the present invention designates biological material which has been removed from its original environment (the environment in which it is naturally located).

For example, a polynucleotide present in the natural state in a plant or an animal is not isolated.

The same polynucleotide separated from the adjacent nucleic acids within which it is naturally inserted in the genome of the plant or the animal is isolated.

Such a polynucleotide may be included in a vector and/or such a polynucleotide may be included in a composition and remain nonetheless in the isolated state owing to the fact that the vector or the composition does not constitute its natural environment.

The term "purified" does not require that the material be present in an absolutely pure form, exclusive of the presence of other substances. It is rather a relative definition.

A polynucleotide is in the purified state after purification from the starting material or from the natural material by at least one order of magnitude, preferably two or three orders of magnitude and even more preferably four or five orders of magnitude.

For the purposes of the present description the expression "nucleotide sequence" can be used to designate indiscriminately a polynucleotide or a nucleic acid. The expression "nucleotide sequence" includes the genetic material itself and is hence not limited to information concerning its sequence.

The invention also relates to a nucleic acid coding for the two protein subunits of the coenzyme B12-independent glycerol dehydratase of *Clostridium butyricum.*

Preferably, such a nucleic acid is characterized in that it comprises a first polynucleotide having at least 50 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 1 and a second polynucleotide having at least 50 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 2.

According to a particular embodiment, such a nucleic acid coding for the two protein subunits of the coenzyme B12-independent glycerol dehydratase should comprise in addition a sequence with a promoter function, functional in the host cell in which the expression of the polynucleotides coding for the two subunits of this enzyme is desired.

Such a transcription promoter nucleotide sequence may be the promoter sequence SEQ ID No. 3 or a sequence having at least 80 % nucleotide identity with this latter.

Another object of the invention is a nucleic acid with a bacterial promoter function, in particular in *Clostridium butyricum*, comprising a polynucleotide having at least 80 % nucleotide identity with the sequence SEQ ID No. 3, or a polynucleotide with a complementary sequence.

As already mentioned above, organization in a unique operon makes possible, in the bacterium *Clostridium butyricum,* the synthesis of a transcription product (messenger RNA) comprising both the sequences coding for the two protein subunits of the coenzyme B12-independent glycerol dehydratase, which catalyses the conversion of glycerol to 3-hydroxypropionaldehyde (3-HPA) and the 1,3-propanediol dehydrogenase (DHAT) which catalyses the conversion of 3-HPA to 1,3-propanediol.

The invention thus also relates to a nucleic acid comprising all or part of a polynucleotide coding for a 1,3-propanediol dehydrogenase having

at least 90 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 4, or a complementary polynucleotide sequence.

In a particular embodiment of a purified or isolated nucleic acid according to the invention, it may be advantageous to be able to include in the same sequence the nucleic acids coding respectively for the two protein subunits of the glycerol dehydrogenase and for the 1,3-propanediol dehydrogenase, such a nucleic acid coding then for the two enzymes capable of catalysing all of the bioconversion steps from glycerol to 1,3-propanediol.

Consequently, the invention also relates to a nucleic acid comprising from the 5' end to the 3' end :

a) a first nucleic acid characterized in that it comprises a first polynucleotide having at least 50 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 1 and a second polynucleotide having at least 50 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 2 ;

b) a second nucleic acid comprising a polynucleotide coding for a 1,3-propanediol dehydrogenase having at least 90 % nucleotide identity with the polynucleotide of sequence SEQ ID No. 4.

The nucleotide sequences coding respectively for the glycerol dehydratase and the 1,3-propanediol dehydrogenase of the nucleic acid described above can in addition also be advantageously placed under the control of a suitable promoter sequence, such as the sequence SEQ ID No.3 or any other promoter sequence functional in the host cell in which their expression is desired.

A nucleic acid complying with the above definition is for example the polynucleotide of sequence SEQ ID No.5 or also a polynucleotide having at least 50 % nucleotide identity with the polynucleotide sequence SEQ ID No.5.

The nucleic acid sequence SEQ ID No.5 comprises the following characteristic functional elements :

a) a transcription terminator for the coding region located upstream from the 1,3-propanediol operon in the *Clostridium butyricum* genome.

This transcription terminator motif possesses a hairpin structure centred on the nucleotide at position 27 of the sequence SEQ ID No. 5 (ΔG = -25.2 kcal/mol) which comprises a 19 bp stem, the two strands of the stem being constituted respectively by the nucleotides in positions 5 to 23 and nucleotides in positions 30 to 48 of the sequence SEQ ID No. 5, the loop of the hairpin structure being constituted by the nucleotides in positions 24 to 29 of the sequence SEQ ID No. 5.

This transcription terminator motif is followed by the sequence ATTTT.

b) Promoter of the 1,3-propanediol operon.

The promoter of the 1,3-propanediol operon is located between the nucleotide at position 100 and the nucleotide at position 200 of the sequence SEQ ID No. 5.

This promoter comprises a TAGATA box sequence (-35) located between the nucleotide at position 142 and the nucleotide at position 147 of the sequence SEQ ID No.5. This promoter also comprises a TATTAT box sequence (-10) located between the nucleotide at position 164 to the nucleotide at position 169 of the sequence SEQ ID No. 5, the distance between the –35 boxes and the –10 boxes being 16 bp.

c) *orf11*(sequence coding for the first subunit of the glycerol dehydrogenase).

It is a unique open reading frame of 2,361 bp coding for the ORF11 polypeptide of 787 amino acids of sequence SEQ ID No. 6.

The initiation codon ATG is located between the nucleotide at position 313 and the nucleotide at position 315 of the sequence SEQ ID No. 5. The open reading frame is terminated by a stop codon with the sequence TAA located between the nucleotide at position 2674 and the nucleotide at position 2676 of the sequence SEQ ID No. 5.

In addition, a ribosomal binding site with the sequence GAGGAG precedes the initiation codon and is located between the nucleotide at position 302 and the nucleotide at position 307 of the sequence SEQ ID No.5.

d) *orf12* (sequence coding for the second subunit of the glycerol dehydrogenase). It is a unique open reading frame of 912 bp coding for a polypeptide of 304 amino acids of sequence SEQ ID No. 7. The initiation

codon with the sequence ATG is located between the nucleotide at position 2704 and the nucleotide at position 2706 of the sequence SEQ ID No. 5. The open reading frame is terminated by a stop codon with the sequence TAA located between the nucleotide at position 3616 and the nucleotide at position 3618 of the sequence SEQ ID No. 5.

In addition, a ribosomal binding site with the sequence AAGGGGA precedes the initiation codon and is located between the nucleotide at position 2689 and the nucleotide at position 2695 of the sequence SEQ ID No. 5.

e) *dhat* (sequence coding for 1,3-propanediol dehydrogenase)

It is a unique open reading frame of 1155 bp coding for a polypeptide of 385 amino acids of the sequence SEQ ID No.8.

The initiation codon with the sequence ATG is located between the nucleotide at position 3678 and the nucleotide at position 3680 of the sequence SEQ ID No.5.

The open reading frame is terminated by a stop codon with the sequence TAA located between the nucleotide at position 4833 and the nucleotide at position 4835 of the sequence SEQ ID No.5.

In addition, a ribosomal binding site with the sequence AGGAGA precedes the initiation codon and is located between the nucleotide at position 3663 and the nucleotide at position 3668 of the sequence SEQ ID No.5.

f) Transcription terminator of the 1,3-propanediol operon.

The transcription terminator of the 1,3-propanediol operon possesses a hairpin structure centred on the nucleotide at position 4933 of the sequence SEQ ID No.5 (ΔG = -27.4 kcal/mol) and comprises a stem of 22 bp constituted respectively by the nucleotides located at positions 4909 to 4930 of the sequence SEQ ID No.5 and the nucleotides located at positions 4936 to 4957 of the sequence SEQ ID No.5.

The loop of the hairpin is constituted by the sequence extending from the nucleotide at position 4931 to the nucleotide at position 4935 of the sequence SEQ ID No.5.

The hairpin structure is followed by the sequence TATTTTAATT.

Each of the functional sequences comprised in the 1,3-propanediol operon of sequence SEQ ID No.5 such as described above may be

employed individually, for example by insertion in a cloning and/or expression vector irrespective of whether it is one of the regions coding for a polypeptide of the invention or a regulatory region (transcription promoter or terminator).

These nucleotide sequences of interest can also be obtained according to the procedures well-known to the man skilled in the art such as the use of restriction enzymes, the use of which is described in detail in the monograph by **SAMBROOK et al.** (1989) or also by selective amplification of the target sequence of interest, for example by PCR.

Also included in the invention are the nucleic acids hybridizing under hybridization conditions of high stringency with a nucleic acid selected from the nucleotide sequences SEQ ID No.1 to SEQ ID No.5.

By "hybridization conditions of high stringency" in the sense of the present invention is meant the following hybridization conditions :

- prehybridization of the filters for 8 hours at 65°C in a buffer composed of 6 x SSC, 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 0.02 % PVP, 0.02 % FICOLL, 0.02 % SAB and 500 µg/ml of denatured salmon sperm DNA ;
- hybridization of the filters for 48 hours at 65°C in the presence of 1 x SSC buffer corresponding to 0.15 M of NaCl and 0.05 M of sodium citrate ;
- three washes of the filter in a solution containing 2 x SSC buffer and 0.1 % SDS at 68°C for 15'.

The high stringency conditions defined above are adapted to the hybridization of a nucleic acid molecule 20 nucleotides long.

It is obvious that these hybridization conditions must be adapted as a function of the length of the nucleic acid whose hybridization is required, in accordance with procedures well-known to the man skilled in the art.

The conditions suitable for hybridization may for example be adapted according to the teaching contained in the monograph of **HAMES & HIGGINS** (1985) or also in the monograph of **SAMBROOK et al. ;** (1989).

Also included in the invention are the nucleic acids comprising at least 20 consecutive nucleotides of a polynucleotide selected from the nucleotide sequences SEQ ID No.1 to SEQ ID No.5.

Such nucleic acids advantageously comprise 20, 25, 30, 35, 40, 50, 100, 150, 200 to 250, 300, 400, 500 consecutive nucleotides of a polynucleotide selected from the nucleotide sequences SEQ ID No.1 to SEQ ID No.5.

Such nucleic acids may comprise 20, 25, 30, 35, 40, 50, 100, 150, 200, 250, 300, 400 or 500 consecutive nucleotides of a polynucleotide selected from the nucleotide sequences SEQ ID No.1 to SEQ ID No.5.

Such nucleic acids may in particular be useful as nucleotide probes or primers in order to detect the presence of any one of the nucleotide sequences of SEQ ID No.1 to SEQ ID No.5 in a sample.

Such nucleotide probes or primers can be used in particular in order to measure the expression of any one of the transcription products of the coding regions *orf11*, *orf12* or *dhat* in accordance with the procedures well-known to the man skilled in the art.

In order to improve still further the capacity of the host cells transformed with a nucleic acid according to the invention to produce 1,3-propanediol from glucose, such a recombinant cell host can also be transformed with one or more genes coding for one or more enzymes capable of catalysing the transformation of glucose to glycerol.

An enzyme couple capable of carrying out the conversion of glucose to glycerol is for example a glycerol-3-phosphate dehydrogenase and a glycerol-3-phosphatase.

Thus, a nucleic acid according to the invention will comprise, in addition to the sequences coding for the coenzyme B12-independent glycerol dehydratase and the 1,3-propanediol dehydrogenase (dhaT), a third nucleic acid coding for a glycerol-3-phosphate dehydrogenase and a fourth nucleic acid coding for a glycerol-3-phosphatase.

In particular, it will be possible to use a nucleic acid coding for gpd1 glycerol-3-phosphate dehydrogenase and a fourth nucleic acid coding for gpp2 glycerol-3-phosphatase.

Gpd1 is described for example by **LARSSON et al.** (1993) Mol. Microbiol., 10, 1101-1111.

Gpd2 is described for example by **HIRAYAMA et al.** (1995) . Mol. Gen. Genet., 249, 127-138.

In accordance with yet another aspect, the invention relates to a recombinant cloning and/or expression vector comprising a nucleic acid coding for a coenzyme B12-independent glycerol dehydratase or a 1,3-propanediol dehydrogenase according to the invention.

Such a recombinant vector will advantageously comprise a constitutive or inducible promoter sequence capable of directing the expression of the coenzyme B12-independent glycerol dehydratase and/or a 1,3-propanediol dehydrogenase and a Rho-independent transcription terminator.

It may, for example, be a shuttle vector capable of replicating in different cell hosts.

A first preferred recombinant vector according to the invention is the plasmid pSPD5 contained in the *Escherichia coli* strain filed at the National Collection of Cultures of Micro-organisms (NCCM) on 24 June 1999 under the access No. I-2243.

Other preferred vectors according to the invention are, for example, the following :

- the vectors pTPG(-) and pOPG represented in Figures 3 and 4.
- the vectors pSGD and pPPF2 represented in Figures 5 and 6 ;
- vectors possessing the replicon of pCB101, such as the vector pCTC511 (**WILLIAMS et al.**, 1990) or also the vector pSYSL2 (**LEE et al.**, 1992)
- a shuttle vector carrying the replicon pAMβ1 of *Enterococcus faecalis* DS-5, such as the vector pCTC41 (**WILLIAMS et al.**, 1990) ;
- the *E. coli B subtilis/C. acetobutylicum* shuttle vectors designated pKNT11 and pKNT14 (**TRUFFAUT et al.**, 1989) ;

The invention also relates to a recombinant host cell comprising a nucleic acid or recombinant vector according to the invention.

Such a recombinant host cell advantageously comprise a nucleic acid coding for the coenzyme B12-independent glycerol dehydratase or also a vector containing such a nucleic acid.

Preferably, such a recombinant host cell should comprise a nucleic acid coding both for the two protein subunits of the coenzyme B12-independent glycerol dehydratase as well as for the DHAT.

It may be, indiscriminately, a bacterium, a fungus or a yeast.

A recombinant bacterial host cell will be preferably selected from *Escherichia coli, Clostridium* or also *Bacillus, Lactobacillus* and *Lactococcus.*

A recombinant yeast cell according to the invention will preferably be the *Saccharomyces cerevisiae* strain.

A preferred recombinant host cell according to the invention is the *Escherichia coli* strain filed at the National Collection of Cultures of Micro-organisms (NCCM) on 24 June 1999 under the access No. I-2243.

The object of the invention also includes the polypeptides constituting respectively each of the two protein subunits constituting the dimeric coenzyme B12-independent glycerol dehydratase according to the invention.

Preferably, a polypeptide according to the invention is available in an isolated or purified form.

The invention also relates to a polypeptide constituting the enzyme 1,3-propanediol dehydrogenase of *Clostridium butyricum.*

More particularly, the invention relates to a polypeptide comprising all or part of an amino acid sequence having at least 50 % amino acid identity with the sequence SEQ ID No.6 or SEQ ID No.7.

The invention also relates to a dimeric protein composed of a first polypeptide having at least 50 % amino acid identity with the polypeptide of sequence SEQ ID No.6 and a second polypeptide having at least 50 % amino acid identity with the polypeptide of sequence SEQ ID No.7.

In a much preferred manner, such a polypeptide exhibits a glycerol dehydratase catalytic activity not requiring the presence of coenzyme B12, such a catalytic activity being capable of being measured in conformity with the examples.

Another object of the invention consists of a polypeptide comprising all or part of an amino acid sequence having at least 80 % amino acid identity with the sequence SEQ ID No.8.

In a very preferred manner, such a polypeptide exhibits a catalytic activity of 1,3-propanediol dehydrogenase.

The invention also relates to a process for the production of a polypeptide according to the invention, characterized in that it comprises the following steps :

a) preparation of a recombinant expression vector according to the invention ;

b) introduction of the recombinant expression vector of step a) into a suitable host cell ;

c) culture of the recombinant host cell of step b) in a suitable culture medium ;

d) recovery of the recombinant polypeptide produced from the culture supernatant or from the cell lysate ;

e) if necessary, purification of the polypeptide recovered.

The polypeptides according to the invention can be purified for example by passage through a nickel or copper ion affinity chromatography column.

These polypeptides can be characterized in addition by their glycerol dehydratase or 1,3-propanediol dehydrogenase enzymatic activity, as indicated in the examples.

The polypeptides according to the invention can also be purified for example by high performance liquid chromatography such as reverse phase and/or cation exchange HPLC chromatographies.

Also included in the invention are the polypeptides comprising amino acid modifications ranging from 1, 2, 3, 4, 5, 10 to 20 substitutions, additions or deletions of an amino acid with respect to the sequence of one or other of the two protein subunits of the coenzyme B12-independent glycerol dehydrogenase or of the 1,3-propanediol dehydrogenase.

In a very preferred manner, the amino acid modifications in the polypeptide of the invention relative to the reference polypeptides should not induce a significant change in their biological activity. Thus, the modifications in the amino acid sequence of the protein subunits of the coenzyme B12-independent glycerol dehydratase according to the invention should be such that the catalytic activity will be at least equal to 50 % of the

initial catalytic activity and will preferably be an improvement on the initial catalytic activity.

The same holds for the amino acid modifications in the protein sequence of the 1,3-propanediol dehydrogenase according to the invention.

Any one of the polypeptide according to the invention or even a peptide fragment of the latter can be used for the preparation of antibodies directed specifically against this latter.

An object of the invention is also constituted by a polypeptide comprising at least 20 consecutive amino acids of an amino acid sequence selected from the sequences SEQ ID No. 6 to SEQ ID No. 8.

Advantageously, such a polypeptide should comprise 20, 25, 30, 35, 40, 45, 50, 75 to 100, 125, 150 or 200 consecutive amino acids of a polypeptide selected from the sequences SEQ ID No. 6 to SEQ ID No. 8.

Preferably, such a polypeptide should comprise 20, 25, 30, 35, 40, 45, 50, 60, 70, 80, 90, 100, 125, 150, 175, 200, 250 or 300 consecutive amino acids of a polypeptide selected from the amino acid sequences SEQ ID No. 6 to SEQ ID No. 8.

Such specific antibodies can be used in immunodetection assays making it possible to determine the presence of a coenzyme B12-independent glycerol dehydratase or of a 1,3-propanediol dehydrogenase in a sample.

Such an immunodetection assay represents an alternative to the determination of glycerol dehydratase or 1,3-propanediol dehydrogenase activity in a sample suspected of containing these enzymes.

By "antibodies" in the sense of the invention, is meant polyclonal antibodies but also monoclonal antibodies such as those prepared from hybridomas according to the procedure described by **KOHLER & MILSTEIN** (1975).

Antibodies in the sense of the present invention also include antibody fragments (Fab', F(ab')2 as well as simple chain FV antibody fragments (US patent No. 4, 946,778 ; **MARTINEAU et al.**, (1998)) or also humanised antibodies (**REINMANN et al.**, 1997 ; **LEGER et al.** 1997).

The invention will in addition be illustrated by the following figures and examples without being limited by them.

Figure 1 represents a schema of the metabolic bioconversion route of glycerol to 1,3-propanediol in the bacterium *Clostridium butyricum*.

Figure 2 illustrates the construction of the vector pSPD5

Figure 3 illustrates the construction of the vector pTPG (-)

Figure 4 illustrates the construction of the vector pOPG

Figure 5 illustrates the construction of the vector pSGD

Figure 6 illustrates the construction of the vector pPPD2

Figure 7 illustrates the production of 1,3-propanediol from glycerol by the *Clostridium acetobutylicum* strain DG1 (pSPD5).

Figure 8 illustrates the production of 1,3-propanediol from glycerol by the *E. coli* strain DH5α transformed by the vector pSPD5 and grown under anaerobiosis.

Figure 9 illustrates the production of 1,3-propanediol from glycerol by the non-recombinant *Clostridium butyricum* strain VPI 1718.

Figure 10 illustrates the production of 1,3-propanediol from glucose by the *Clostridium acetobutylicum* strain DG1[pSPD5].

MATERIALS AND METHODS

I. CULTURE OF THE STRAINS

1.Strain used

The *Clostridium butyricum* strain from which the different nucleic acid sequences according to the invention were isolated and characterized is the strain VPI 1718.

The *Clostridium acetobutylicum* strain used for transformation with the different vectors described in the examples is the strain available from the American Type Culture Collection under the No. ATCC 824.

The *Escherichia coli* strain DH5α used for the transfection with the different plasmids described in the examples is the strain available from LIFE TECHNOLOGIES Inc.

2.The plasmids

The 2.7 kb plasmid pUC18 is described by **YANNISCH-PERRON et al.** (1985)

The 4.9 kb plasmid pIMPI is described by **MERMELSTEIN** (1992).

The 7 kb plasmid pSOS95 is described by **SOUCAILLE** and **PAPOUTSAKIS** (1996).

3. Culture media and culture conditions

For the culture of the *Escherichia coli* strain the LB medium (described in **SAMBROOK et al.**, 1989) is supplemented with antibiotics (100 µg/ml ampicillin, 300 µg/ml erythromycin and 35 µg/ml chloramphenicol).

The cultures of the *Clostridium* strains, in particular the *Clostridium butyricum* strains or *Clostridium acetobutylicum* DGI strains transformed by the vector pSPD5 were carried out in the following medium, the composition of which is given for 1 liter of culture medium :

Yeast extract	4 g
Glycerol	60 g
KH_2PO_4	0.5 g
K_2HPO_4	0.5 g
$MgSO_4$	0.2 g
NH_4Cl	1.5 g
$FeSO_4$	10 mg
Biotin	0.04 mg
Para-aminobenzoic acid	8 mg

The pH is adjusted to 6 by addition of ammonium and the growth temperature is 37°C.

Clarithromycin is added at a concentration of 40 mg/l for the culture of the *C. acetobutylicum* D61 (pSPP5) strain.

II. ANALYTICAL TECHNIQUES

1. Determination of the substrates and products

All of the substrates (glucose and glycerol) and products (acetate, butyrate, lactate, 1,3-propanediol) are determined by means of high performance liquid chromatography (HPLC).

The HPLC apparatus (pump model 5810, Waters) is equipped with an automatic sample changer (SP 8775, Spectra Physic) having a 20 µl injection loop, an integrator (Intersmat ICR 1B, Shimadzu) and a refractometer (HP 1047A).

The separation is obtained by passage through an ion exclusion column (Aminex R HPX-87H, 300 mm x 7.8 mm, Biorad) equipped with a pre-column (Micro-Guard, Biorad) filled with the same ionic resin H^+.

The mobile phase is constituted by 0.031 mM sulfuric acid, the flow rate is 0.7 ml/min and the separation is made at room temperature.

2. Determination of activities

a) Determination of 1,3-propanediol dehydrogenase

The determination of 1,3-propanediol dehydrogenase was carried out in a 1 ml volume of the following reaction medium :

1,3-propanediol	100 mM
NAD^+	2 mM
DTT	2 mM
$(NH_4)_2SO_4$	30 mM
K_2CO_3	100 mM

Cell extract : assays on 5 µl, 10 µl, 50 µl

Made up to 1 ml with water

The reduction of NAD^+ is monitored by the measurement of the optical density at the wavelength of 340 nanometers.

The extinction coefficient ε (NADH) is 6.22 $mM^{-1} \times cm^{-1}$

b) Determination of glycerol dehydratase

The determination of glycerol dehydratase is carried out on a cell extract that has previously passed through a desalting column. This determination is carried out in a 1 ml volume of the following reaction medium :

KCl	0.05 M
1,2-propanediol	0.06 M
KPO_4 buffer, pH7	0.035 M

Cell extract assay on 5 µl, 10 µl, 20 µl, 30 µl

Made up with water to 1 ml

The reaction is stopped after 10 minutes at 37°C with the aid of 1 ml of 100 mM citrate buffer, pH 3.6 and 500 µl of 0.1 % MBTH.

After 15 minutes at 37°C, 1 ml of water is added and the amount of propionaldehyde formed is determined by measurement of the optical density at the wavelength of 305 nanometers.

The molar extinction coefficient of the product formed is 13.3 x 10^3 $M^{-1} \times cm^{-1}$.

c) Measurement of biomass

Bacterial growth is monitored by the measurement in aliquot fractions sampled at defined times of the optical density at the wavelength of 620 nanometers for an optical path of 1 cm. It is considered that one optical density unit corresponds to approximately 3 x 10^8 bacteria/ml.

III. Transformation of the bacteria with the recombinant vectors according to the invention.

III.1. Transformation of E. coli DH5α

The *E. coli* DH5α strain is made competent according to the protocol established by **INOUE et al.** (1990). This protocol makes it possible to obtain transformation efficiencies of the order of 10^8 to 10^9 transformants/mg of pUC18. The cells made competent are then stored at -80°C. The transformation of the competent cells by a plasmid is performed by thermal shock (**SAMBROOK et al.**, 1989).

III. 2. Transformation of C. acetobutylicum

The plasmid to be introduced into *C. acetobutylicum* (ATCC 824 or DG1) must first be methylated at the Cac8241 sites (= Bsofl). The methylation *in vivo* is carried out by introducing the plasmid to be methylated in the *E. coli* ER 2275 strain carrying the plasmid pAN1 (which contains the gene coding for the methylase of the phage f3TI of *Bacillus subtilis*). The plasmid DNA preparation used to transform *C. acetobutylicum* must be very pure, purified by ultracentrifugation in a caesium chloride gradient (SAMBROOK et al., 1989) or by using the Qiafilter Plasmid Midiprep kit (QIAGEN).

The transformations are performed in strict anaerobiosis in accordance with the following protocol, adapted from that of **MEMMERLSTEIN** (1992). The efficiencies are still very low, of the order of 10^2 transformants per mg of DNA.

From an overnight preculture in CGM medium (10 ml) at 37°C

Inoculate 50 ml of 2YTG medium with a 10 % inoculum

Stop the culture at an O.D.600 of 1.0 to 1.2.

From this point onwards all of the operations are carried out in the anaerobic hood and in ice.

Centrifuge the cells for 10 min at 4 000 g

Wash the cell pellet in 10 ml of electroporation buffer.

Centrifuge for 10 min at 3 000-4 000 g.

Resuspend the cells in 500 ml of electroporation buffer.

The cell suspension is placed in contact with the plasmid (5 to 10 mg of plasmid DNA dissolved in 5 to 50 ml of TE buffer) previously introduced into the electroporation cuvette (0.4 cm thickness). The cuvette and contents are stored in ice.

The mixture is immediately subjected to an electric discharge with the following parameters : V = 2500V, C = 25 mF and R infinity (BioRad Gene pulser II and Gene controller II). Under these conditions, the time of delivery of the discharge varies from 7 to 12 ms.

The cells are then immediately transferred to 10 ml of 2 YTG medium and incubated at 37°C until metabolism resumes (formation of bubbles of carbon dioxide and hydrogen).

The culture is then centrifuged and the cell pellet is taken up in a minimal volume of the same medium. The suspension is then spread on RCA medium with an antibiotic.

Composition of the electroporation buffer :

Sucrose	270 mM
Phosphate buffer (Na_2HPO_4/NaH_2PO_4), pH 7.4	3 mM

EXAMPLE 1 : Construction of the expression vector pSPD5

The following nucleotide primers were synthesized in order to amplify a nucleotide sequence containing simultaneously *orf11*, *orf12* and *dhaT*, i.e. all of the sequences coding both for the two protein subunits of the coenzyme B12-independent glycerol dehydratase and the 1,3-propanediol dehydrogenase of *Clostridium butyricum*.

The primer PDH3 (SEQ ID No.9) includes the BamHI site, the ribosomal binding site and the beginning of *orf11*.

The primer PDH4 (SEQ ID No.10) hybridizes with the complementary strand and contains the SmaI site and the end of the dhaT gene.

An amplification reaction with the primers pDH3 and pDH4 was carried out on the genomic DNA of *Clostridium butyricum* under the following conditions :

- annealing temperature for the primers : 55°C ;
- duration of elongation : 4 minutes ;

- number of cycles : 15 cycles

The amplification reaction was carried out with the Expand Long Template PCR kit (sold by Boehringer).

This reaction made it possible to amplify a fragment of the expected size of 4.6 kb.

This amplified fragment was purified on agarose gel then digested by the enzymes BamHI and SmaI.

In parallel, the 4970 bp BamHI-EheI fragment of the *E.coli/C.acetobutylicum* shuttle vector pSOS95 was purified on agarose gel then ligated to the PCR product of 4.6 kb. The recombinant vector thus constructed, called pSPD5 (Figure 2), makes possible the constitutive expression of the genes *orf11, orf12* and *dhaT* under the control of the promoter of the thiolase gene of *C. acetobutylicum*.

EXAMPLE 2 : Construction of the expression vectors pTPG(-) and pOPG

The vectors pTPG(-) and pOPG are vectors derived from the shuttle vector *E. coli-Clostridium* pTLH1 (**HARRIS et al.**, 1999) which enables an adequate host cell to carry out the direct conversion of glucose to 1,3-propanediol. Basically, they allow the expression of the genes *orf11, orf12* and *dhaT* of *C. butyricum* (1,3-propanediol operon) as well as that of the genes GPD1 and GPD2 of *S. cerevisae* coding respectively for glycerol-3-phosphate dehydrogenase and glycerol-3-phosphatase (artificial operon *gly*, carried by the plasmid pS2 (GELIS et al., 1999), derived from the plasmid pSOS95, which makes possible the conversion of glucose to glycerol in *E. coli* and *C. acetobutylicum*). These two plasmids are distinguished by the organization of these genes in two operons (1,3-propanediol and *gly*) in pTPG(-) and on the same operon in pOPG.

2.1. Construction of pTPG(-)

In a first step, the 4.9 kbp *Sal*I fragment of the pSPD5 plasmid containing the entire 1,3-propanediol operon was purified on agarose gel, then ligated to the shuttle vector pTLH1 digested by *Sal*I in order to obtain the pTLP plasmid of 10.6 kbp.

In parallel, the nucleotide primers OPGLY-D and OPGLY-R were synthesized in order to amplify the entire artificial operon *gly*.

The primer OPGLY-D includes the *Sma*I site as well as the beginning of the artificial operon *gly*. Its sequence is the following :

5'-GTTACCCGGGGCTCCTGCAGCTCGACTTTTTAAC-3'

The primer OPGLY-R hybridizes with the complementary strand and includes the *Sma*I site as well as the end of the artificial operon *gly*. Its sequence is the following :

5'-TTTCACCCGGGAAACAGCTATGACCATGATTACG-3'

An amplification reaction with the primers OPGLY-D and OPGLY-R was carried out on the plasmid pS2 under the following conditions :

- annealing temperature for the primers : 48°C ;
- duration of elongation : 3.5 minutes ;
- number of cycles : 10 cycles

The reaction made it possible to amplify a fragment of the expected size of 2.2 kbp.

This amplified fragment was purified on agarose gel and digested by the enzyme *Sma*I, then ligated to the plasmid pTLP linearised by *Sma*I to furnish the plasmid pTPG(-) of 12.8 kbp (Figure 3).

2.2. Construction of pOPG

In a first step, the 2.2 kbp *Pvu*II-*Pst*I fragment of the plasmid pS2 containing the entire glycerol operon was purified on agarose gel, then ligated to the 5.7 kbp *Sma*I-*Pst*I fragment of the shuttle vector pTLH1 in order to obtain the plasmid pTLG1 of 7.9 kbp.

In parallel, the nucleotide primers dhaT-F and dhaT-R were synthesized in order to amplify the 5' end of the *dha*T gene.

The primer DHAT-F includes the *Bam*HI site as well as the central region of the *dha*T gene. Its sequence is the following :

5'-TTGGATCCAGTATCTATAAATGATCCAATGC-3'

The primer DHAT-R hybridizes with the complementary strand and includes the *Bgl*II site as well as the end of the *dha*T gene. Its sequence is the following :

5'-TTAGATCTTTTAAATAGTATTAATTAATAAGCAGCC-3'

An amplification reaction with the primers dhaT-F and dhaT-R was carried out on the plasmid pSPD5 under the following conditions :

- annealing temperature for the primers : 48°C ;
- duration of elongation : 1 minute ;
- number of cycles : 10 cycles

The reaction made it possible to amplify a fragment of the expected size of 0.65 kbp.

This amplified fragment was purified on agarose gel and digested by the enzymes *Bam*HI and *Bgl*II, then ligated to the plasmid pTLG1 linearised by *Bam*HI to furnish the plasmid pTPG of 8.55 kbp.

In a last step, the 4.0 kbp *Bam*HI-*Sbf*I fragment of the plasmid pSPD5 was purified on agarose gel then ligated to the 8.55 kbp *Bam*HI-*Sbf*I fragment of the plasmid pTPG in order to obtain the plasmid pOPG of 12.55 kbp (Figure 4).

EXAMPLE 3 : Construction of the expression vector pSGD

The vector pSGD is derived from the plasmid pSPD5.

Basically, the vector pSGD expresses functionally *orf11* and *orf12* (coding respectively for the first and second protein subunits of glycerol dehydratase) and contains a deletion in the region coding for DHAT (1,3-propanediol dehydrogenase).

The vector pSPD5 was subjected to digestion by the restriction enzyme *Sbf*I and the 4082 bp fragment purified on agarose gel.

Digestion of the vector pSOS95 was then carried out by means of the restriction enzyme *Pst*I and the 4858 bp fragment was purified on agarose gel.

These two fragments were then ligated in order to obtain the plasmid pSGD.

EXAMPLE 4 : Construction of the expression vector pPPD2

The vector pPPD2 is a vector derived from the plasmid pSPD5 capable of expressing the *dhat* gene (coding for the 1,3-propanediol dehydrogenase) and in which *orf11* (coding for the first subunit of the glycerol dehydratase has been entirely deleted as well as 100 bp at the 5' end of *orf12* (coding for the second subunit of the glycerol dehydratase).

The vector pSPD5 was first digested simultaneously by the restriction enzymes *Bam*HI and *Mfe*I.

The 6326 bp *Bam*HI-*Mfe*I fragment obtained by double digestion with the aid of the corresponding restriction endonucleases was subjected to a treatment in the presence of the T4 DNA polymerase in order to obtain blunt ends.

The fragment was then subjected to religation on itself in order to obtain the plasmid pPPD2.

EXAMPLE 5 : Expression of the 1,3-propanediol operon in *Clostridium acetobutylicum* DG1 grown on glycerol

The *Clostridium acetobutylicum* DGI strain transformed with the plasmid pSPD5 as described in the Materials and Methods section was placed in culture in the presence of glycerol for defined times and different parameters were monitored during fermentation :

- bacterial growth was monitored by measurement of the optical density at the wavelength of 620 nanometers and is shown on Figure 7 by the open circles ;

- the glycerol concentration was monitored throughout the fermentation and is shown in Figure 7 by filled squares ;

- the synthesis of 1,3-propanediol was also monitored throughout the fermentation and is shown by filled circles ;

- similarly the acetate and butyrate concentrations were measured throughout fermentation and are shown in Figure 7 by triangles and inverted triangles, respectively.

The results shown in Figure 7 show that a significant quantity of 1,3-propanediol is synthesized by the *Clostridium acetobutylicum* DGI strain transformed by the plasmid pSPD5 after the first four hours of culture. After 20 hours of fermentation, it is possible to observe the production of 38 g/l of 1,3-propanediol. The production of 1,3-propanediol

reaches a plateau about 18 hours after the beginning of fermentation, this plateau of production being essentially due to the fact that almost all of the initial glycerol has been consumed by the transformed bacterium.

It is important to note that the *C. acetobutylicum* DG1 strain transformed by the control plasmid pIMP1 not containing the regions coding for the glycerol dehydratase and the 1,3-propanediol dehydrogenase (**Memmerlstein**, 1992) does not grow on the culture medium with glycerol as sole carbon source and hence does not produce 1,3-propanediol (Table 1).

EXAMPLE 6 : Expression of the 1,3-propanediol operon in Escherichia coli DH5α grown on glycerol.

An *Escherichia coli* DH5α strain was transformed with the vector pSPD5 as described in the Materials and Methods section.

The *Escherichia coli* strain transformed with the plasmid pSPD5 was grown in anaerobiosis on LB medium supplemented with glycerol (40 g/l) and erythromycin (300 µg/ml), the production of 1,3-propanediol was measured at defined times.

The results are summarized in Figure 8.

The results of Figure 8 show that a significant amount of 1,3-propanediol is produced right from the start of fermentation. After 80 hours of fermentation, a production of about 4.5 g/l of 1,3-propanediol can be observed.

The control strain *E. coli* DH5α[pIMP1] grown on the same medium does not lead to the production of 1,3-propanediol (Table 1).

TABLE 1

Production of 1,3-propanediol by recombinant strains grown at pH 6.5 in the presence of glycerol under anaerobic conditions

	1,3-propanediol (g/l)
E. coli DH5α (pIMP1)	0
E. coli DH5α (pSPD5)	4,5
C. acetobutylicum DG1 (pIMP1)	No growth
C. acetobutylicum DG1 (pSPD5)	38

The results of Table 1 show that a significant production of 1,3-propanediol (about 5 g/l) is obtained with the *Escherichia coli* DH5α strain

transfected by the plasmid pSPD5, whereas the *E. coli* strain transfected with the control plasmid pIMP1 does not produce a detectable quantity of 1,3-propanediol.

With the construction pSPD5, a quantity of 1,3- propanediol is observed with the *Clostridium acetobutylicum* strain approximately 7.6 fold higher than with the *Escherichia coli* DH5α strain. This is mainly due to the fact that the regulatory signals of the plasmid pSPD5 were optimised for the expression of the 1,3-propanediol operon in *Clostridium acetobutylicum*.

Regulatory signals such as a very active promoter in *Escherichia coli* are capable of making possible the production of as large a quantity of 1,3-propanediol in *Escherichia coli* as that observed in *Clostridium acetobutylicum*.

EXAMPLE 7 : Expression of the 1,3-propanediol operon in wild-type *Clostridium butyricum* grown on glycerol.

The *Clostridium butyricum* strain VPI 1718 was grown in the presence of glycerol and the synthesis of 1,3-propanediol was measured at defined times throughout fermentation.

The results are presented in Figure 9.

The results of Figure 9 show that the production of 34 g/l of 1,3-propanediol is observed after 60 hours of fermentation.

If reference is made to the results shown in Figure 7, it can be observed that the production of 1,3-propanediol by the *Clostridium acetobutylicum* DG1 strain transformed by the plasmid pSPD5 is much more rapid (22 hours as opposed to 60 hours) and higher than the production of 1,3-propanediol obtained with the *Clostridium butyricum* strain naturally expressing the coenzyme B12-independent glycerol dehydratase and the 1,3-propanediol dehydrogenase according to the invention.

Moreover, the levels of catalytic activity, expressed in units per mg, of glycerol dehydratase and 1,3-propanediol dehydrogenase were measured in both *Clostridium acetobutylicum* and *Clostridium butyricum*.

The results are shown in the DG1[pSPD5] Table 2 below

TABLE 2

Levels of enzymes implicated in the production of 1,3-propanediol in the recombinant *C. acetobutylicum* strains and *C. butyricum*.

	Glycerol dehydratase (U/mg)	1,3-propanediol dehydrogenase (U/mg)
C.acetobutylicum **DG1 (PSPD5)** **Growth on glycerol**	3,5	2,4
C.acetobutylicum **DG1 (pIMP1)** **Growth on glucose**	0,01<	0,005<
C.butyricum **Growth on glycerol**	0,45	0,56

The results presented in Table 2 indicate that the levels of activity of the glycerol dehydratase and the 1,3-propanediol dehydrogenase expressed in the *Clostridium acetobutylicum* strain transformed by the plasmid pSPD5 are much higher (7.8 and 4.3 fold higher, respectively) than the levels of glycerol dehydratase and 1,3-propanediol dehydrogenase activity expressed by the *Clostridium butyricum* strain naturally producing these enzymes.

As control, a *Clostridium acetobutylicum* strain transformed with the plasmid pIMPI not containing the regions coding for the glycerol dehydratase and the 1,3-propanediol dehydrogenase does not produce detectable quantities of these two enzymes, which shows clearly that the activity observed in the strain transformed by the plasmid pSPD5 is solely due to the expression of the 1,3-propanediol operon according to the invention.

The results presented in Table 2 clearly demonstrate the value of transforming a strain of micro-organism not naturally producing glycerol dehydratase and 1,3-propanediol dehydrogenase according to the invention with a recombinant vector such as the vector pSPD5 because it is possible

in this case for example by the use of appropriate promoters to obtain levels of expression which are never attained naturally.

EXAMPLE 8 : expression of the genes of the 1,3-propanediol operon in Saccharomyces cerevisia

A. Materials and Methods

A.1 Materials

MSL MEDIUM	
SALTS	g/l
- $(NH_4)_2SO_4$	5
- $MgSO_4$. $7H_2O$	0.5
- KH_2PO_4	1
- NaCl	0.1
-- $CaCl_2$. $2H_2O$	0.1

VITAMINS	**mg/l**
- d-biotin	0.02
- Ca D(+) pantothenate	0.4
- myo-inositol	2.0
- thiamine HCl	0.4
- pyridoxine HCl	0.4
- p-aminobenzoic acid	0.2
- folic acid	0.02
- niacin	0.4
- riboflavin	0.2

TRACE ELEMENTS	**mg/l**
- $ZnSO_4$. $7H_2O$	0.4
- $MnSO_4$	0.4
- $CuSO_4$. $5H_2O$	0.04
- Na_2MoO_4. $2H_2O$	0.2
- $FeCl_3$. $6H_2O$	0.2
- H_3BO_3	0.5
KI	0.1

CARBON SOURCE	g/l
- glucose	10
- glycerol	10

AA (drop-out)	g/l
- L-Arg	0.02
- Thre	0.05
- L-Tryp	0.04
- L-Isoleu	0.06
- Lys	0.04
- Met	0.01
- Phe	0.06
- Tyr	0.05
- Adenine	0.01
- Ergosterol	0.01
- Tween 80	0.42

A.2. Methods

The genes *orf11, orf12* and *dhat* were individually amplified by means of PCR after introducing a *Sma*I site at the 5' end and an *Apa*I site at the 3' end by the use of primers. Through the agency of these two restriction sites each gene was then introduced into the vector pYGK (CROUX and SOUCAILLE, 1999) making it possible to construct an expression cassette under the control of the promoter of the PGK gene and of the terminator of the CYC1 gene. The three genes in their expression cassette were then cut out from each of the vectors previously obtained (pYGK11, pYGK12 and pYGKT) by *Not*I-*Sac*II digestion and introduced into three multimeric vectors of the family pRS42x which differ by the nature of their selection marker (HIS3 for pRS423, LEU2 for pRS425 and URA3 for pRS426) previously digested by the same restriction enzymes. Each of the three plasmids obtained (pRSGK11, pRSGK12 and pRSGKT) was then introduced into the *S. cerevisae* JF624 (*leu*2, *ura*3 *lys*2 *trp*1 *his*3) strain and selected by complementation with the 3 auxotrophies of this strain. The *S. cerevisae* JF624 (pRSGK11, pRSGK12, pRSGKT) strain and

the control strain *S. cerevisae* JF624 (PRS423, PRS425, pRS426) were then grown in anaerobiosis for 36 hours in the MSL medium.

B. RESULTS

The results of the expression of the genes of the 1,3-propanediol operon in the *S. cerevisae* JF624 strains described in paragraph A.2 above are presented in Table 3 below.

The results in Table 3 show that the *S. cerevisae* JF624 strain which contains the inserts of the genes *orf11, orf12* and *dhat* inserted respectively in the pRSGK11, pRSGK12 and pRSGKT plasmids is capable of producing 1,3-propanediol from a glycerol source without the addition of vitamin B12.

TABLE 3

	Ethanol (g/l)	1,3-propanediol (g/l)
S. cerevisae JF624 (pRS423, pRS425,pRS426	4,5	0
S. cerevisae JF624 (pRSGK11, pRSGK12, pRSGKT)	4,8	0,1

EXAMPLE 9 : Expression of the 1,3-propanediol operon in *Clostridium acetobutylicum* DG1 [pSPD5] grown on glucose

The *Clostridium acetobutylicum* DG1 strain transformed by the plasmid pSPD5 as described in the Materials and Methods section was grown in the presence of glucose as sole carbon source and different parameters were monitored during the fermentation.

The results are presented in Figure 10.

The results in Figure 10 show a low level (0.3 g/l) of synthesis of 1,3-propanediol whereas all of the glucose was consumed. This production, due to the presence of a low concentration of intracellular glycerol in *Clostridium acetobutylicum* DG1 [pSPD5] demonstrates however the feasibility of a direct conversion process of glucose to 1,3-propanediol in a strain carrying the genes claimed by the present invention.

EXAMPLE 10

Expression of the 1,3-propanediol operon in Bacillus subtilis 168 [pSPD5] grown on glycerol.

The *Bacillus subtilis* 168 strain transformed by the plasmid pSPD5 as described in the Materials and Methods section was grown on LB medium + 10 g/l glycerol + 20 mM nitrate + 2 µg/ml erythromycin in anaerobiosis and compared to the same strain transformed by the control plasmid pIMP1.

The results are presented in Table 4 below

TABLE 4

	Acetate (g/l)	2,3-butanediol (g/l)	1,3-propanediol (g/l)
B. subtilis 168 (pSPD5)	0,84	1,2	0
B. subtilis 168 (pSPD5)	0,9	1,0	0,2

The results in Table 3 show that the expression of the 1,3-propanediol operon in *B. subtilis* makes possible the production of 1,3-propanediol from glycerol without the addition of vitamin B12.

EXAMPLE 11 : Conversion of glucose to 1,3-propanediol by *E. coli* NZN 111 [pTPG].

The plasmid pTPG(-) and the control plasmid pTLH1 were introduced into the *E. coli* NZN 111 (pfl :cat, Idh :kan) strain, a strain incapable of producing formate and lactate in anaerobiosis. These two recombinant strains were then grown anaerobically for 48 hours in the LB medium + glucose + 10 µg/ml of tetracycline without regulation of pH. The results presented in Table 5 show that *E. coli* can produce significant quantities of 1,3-propanediol from glucose after transformation by the plasmid pTPG.

TABLE 5

	Acetate g/l	Succinate (g/l)	Glycerol (g/l)	1,3 propanediol (g/l)
E.coli NZN111 (pTLH1)	0,15	0,4	0	0
E.coli NZN111(pTPG)	1,25	0,2	2,1	0,7

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